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The Properties of the Heavy and Light Subunits of Bovine Enterokinase

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This research project was concerned with the structure, function, and physiological properties of bovine enterokinase. The light chain is the catalytic subunit and contains the histidine-serine-aspartic acid of the active site. The catalytic subunit is obtained as a functional subunit after cleavage of the connecting disulfide using mild reducing conditions. We are studying the amino acid sequence and enzymatic properties of the catalytic subunit.

The isolation procedure was improved for bovine enterokinase and for the preparation of the catalytic subunit. These two accomplishments made it possible to obtain sufficient amounts of highly purified enzyme to perform a partial amino-terminal sequence analysis of the light chain and to establish the disulfide content of the chain. The properties of a functional catalytic subunit differed from those found in the past.

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Milder reaction conditions produced a more native form of the enzyme. The action of the enzyme toward small synthetic substrates and toward the substrate trypsinogen and the protein inhibitor bovine pancreatic trypsin inhibitor were investigated. The specificity characteristics suggested that the heavy subunit may be necessary to form a productive enzyme-substrate complex with the large substrate molecules.

FINAL TECHNICAL REPORT

Army Research Office Research Agreement No. DAAL03-86-K-0163

The Properties of the Heavy and Light Subunits of Bovine Enterokinase.

STATEMENT OF PROBLEM

Enterokinase (enteropeptidase) is the proteolytic enzyme responsible for initiating mammalian digestion. The enzyme is known to be the physiological activator of trypsinogen. The trypsin molecules then act in a cascade mechanism to activate other zymogens needed in the intestinal duodenum for the complete hydrolysis of foodstuffs to their monomeric units. Indeed, a known genetic lesion, where individuals lack enterokinase activity, causes severe malnutrition and neurological damage in the newborn. The enzyme has a limited specificity for the sequence of the activation peptide of trypsinogen and this remarkable property has been exploited by researchers in using the enzyme as a tool in cloning proteins.

The only available source for bovine enterokinase is the isolation and purification from the duodenal section of the intestine. Unfortunately, the content of the enzyme is extremely low and a typical laboratory preparation only yields 10 to 12 mg of highly purified enzyme after 10 to 15 days of intensive work. The possibility of repeating the purification again and again is limited by the availability of the intestines. The slaughter of beef has been on the decline since the eating habits of Americans changed a few years ago. As a result, the collection of intestines is the rate-limiting step. The alternative approach of cloning the gene and expressing the protein is not possible until a suitable methodology is developed for bovine enterokinase. We will explore these possibilities in a collaborative venture with the Shemyakin Institute of Bioorganic Chemistry of the USSR Academy of Sciences.

The limiting supply of enzyme is a critical factor in the studies performed in the contract period. The supply of enzyme is crucial because of the very large size of the molecule (150 kdaltons). The structure of the enzyme has a heavy (105 kdaltons) and a light chain (35 kdaltons) covalently linked by a single disulfide bond. Since enterokinase is a member of the serine protease family, its

size and over-all structure appear to resemble the large blood clotting and fibrinolytic proteins more than pancreatic trypsin, chymotrypsin, and elastase. This observation is central to the design and interpretation of the experiments described below. The presence of 35 to 40 per cent carbohydrate in both chains makes enterokinase a glycoprotein that is more unique than other known serine proteases. Furthermore, the carbohydrate content creates experimental difficulties in the purification of the enzyme and in the characterization of its structure.

Our research project is concerned with the structure, function, and physiological properties of bovine enterokinase. The light chain is the catalytic subunit and contains the histidine-serine-aspartic acid of the active site. The catalytic subunit is obtained as a functional subunit after cleavage of the connecting disulfide using mild reducing conditions. We are studying the amino acid sequence and enzymatic properties of the catalytic subunit.

We improved the isolation procedure for bovine enterokinase and for the preparation of the catalytic subunit. These two accomplishments made it possible to obtain sufficient amounts of highly purified enzyme to perform a partial amino-terminal sequence analysis of the light chain and to establish the disulfide content of the chain. The properties of a functional catalytic subunit differed from those found in the past. Milder reaction conditions produced a more native form of the enzyme. The action of the enzyme toward small synthetic substrates and toward the substrate trypsinogen and the protein inhibitor bovine pancreatic trypsin inhibitor were investigated. The specificity characteristics suggested that the heavy subunit may be necessary to form a productive enzyme-substrate complex with the large substrate molecule.

The studies accomplished in this contract period were aided by the development of a new HPLC assay of enterokinase. After trypsinogen was activated to trypsin, a stable complex was immediately produced in the presence of chicken ovomucoid. The complex was easily separated from the substrate on size-exclusion HPLC and the size of the peak was directly related to the amount of

enzyme used in the activation process. The increased sensitivity and greater reliability of the new procedure compared to the classical assay contributed to our success in the study of the catalytic subunit.

A summary of the individual studies performed in the project period are as follows.

A Direct High-Performance Liquid Chromatography Assay of the Enzymatic Activity of Enterokinase

Bovine enterokinase activates trypsinogen to trypsin at pH 8.0. In the presence of chicken ovomucoid, a stable complex of ovomucoid-trypsin is produced, inactivating trypsin and eliminating autoactivation of trypsinogen. The molecular size of trypsin (24,000 Da) is increased twofold on forming the ovomucoid-trypsin complex (52,000 Da). Size-exclusion chromatography on a Toya Soda *TSK G2000SW* column in an HPLC system and with computer-assisted analyses gives a direct quantitative determination of the amount of substrate (trypsinogen) and product (ovomucoid-trypsin). The rate of disappearance of substrate is equal to the rate of formation of product in agreement with kinetic theory. The simultaneous determination of both rates increases the reliability of the assay. The HPLC assay has an extended linear range for the velocity of the activation process as a function of enzyme concentration. The assay is reliable and accurate for highly purified preparations, samples at different steps in the purification scheme, and for a direct assay of the intestinal contents. The assay will also be useful in clinical analyses.

The Amino-Terminal Sequence of the Catalytic Subunit of Bovine Enterokinase.

We reexamined the amino acid composition of the S-carboxymethylated light chain and found nine half-cystine residues. The half-cystine content suggested that the light chain has four intramolecular disulfides (8 half-cystines) and one disulfide (one half-cystine) linking the light and heavy chains. The amino-terminal 27 residues of the S-vinylpyridyl derivative of the light chain were determined by automated Edman degradation. The sequence has homologies with a variety of other

known serine proteases containing one or two chains. The homologies suggest that the catalytic subunit has the same three-dimensional structure, and therefore, the same mechanism of enzymatic action as chymotrypsin, trypsin, and elastase. The conserved amino-terminal activation peptide sequence (IVGG) suggests that enterokinase has a zymogen precursor and that the two-chain enzyme arises from limited proteolysis. The 27-residue sequence has appropriate peptide regions that will be useful for the construction of polynucleotide probes to select a c-DNA from a gene library and a sequence of six to eight residues that could serve as an epitope for the production of antibodies to the enzyme.

The Catalytic Subunit of Bovine Enterokinase. The Peptidase and Esterase Activities.

Conditions were used for the chemical reduction of native bovine enterokinase that limited the cleavage to a single disulfide bond between the light (catalytic subunit) and heavy chain. With these conditions, the four intramolecular disulfide bonds of the catalytic subunit remained intact. The purified light chain had one S-carboxymethyl cysteine residue per molecule of protein. This contrasts with the report of Light and Fonseca (J. Biol. Chem, **259**, 13195-13198 (1984) where the reducing conditions were more drastic and three S-carboxymethyl cysteine residues were found. The catalytic subunit retained normal esterase activity while the activity toward trypsinogen and Gly-$(Asp)_4$-Lys-NA (peptidase activity) was about 10 to 20 percent of the native enzyme. The subunit with three S-carboxymethyl cysteine residues also had normal esterase activity but low peptidase activity. Removal of the heavy subunit may have increased the flexibility of the binding site for peptidase activity while maintaining the esterase activity. We hypothesize that altered enzyme-substrate binding decreased the concentration of a productive ES complex and therefore decreased the peptidase activity.

List of Publications During the Project Period

Light, A. and Janska, H. Enterokinase (Enteropeptidase): Comparative Aspects. TIBS **14**, 110-111, 1989.

Janska, H. and Light, A. A Direct High-Performance Liquid Chromatography Assay of the Enzymatic Activity of Enterokinase (Enteropeptidase). Anal Biochem. **176**, 132-136, 1989.

Light, A. and Pfeifer, R. F. An Introduction to Section V, Protein Chemistry and Analyses in "Techniques in Protein Chemistry", (Hugli, T. E., ed.), Academic Press, p. 417, 1989.

Janska, H. and Light, A. A New Assay for Enterokinase in "Techniques in Protein Chemistry", (Hugli, T. E., ed), Academic Press. pp.490-496, 1989.

Light, A. and Janska, H. The Amino-Terminal Sequence of the Catalytic Subunit of Bovine Enterokinase. Submitted for publication in "Proteins. Structure, Function, and Gentics".

Light, A. and Janska, H. The Catalytic Subunit of Bovine Enterokinase. The Peptidase and Esterase Activities. Manuscript in preparation. Preliminary copy enclosed.

List of Participating Scientific Personnel

Albert Light, Principal Investigator

Hanna Janska, Post-doctoral Fellow

Drew Paar, Undergraduate student

Others - Lab helpers, dishwashers

Grants and Contracts

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